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Amplified expression, purification and functional reconstitution of the dipeptide and tripeptide transport protein of *Lactococcus lactis*

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Transport of hydrophilic dipeptides and tripeptides into *Lactococcus lactis* is mediated by a proton-motive-force-driven peptide-transport protein (DtpT) that shares similarity to eukaryotic peptide transporters, e.g. from yeasts, plants, and the kidney and small intestine of rabbit, man and rat. The expression level of DtpT protein in *L. lactis* was increased (20–40-fold) to approximately 10 % of total integral membrane protein by means of a low-copy-number vector and selecting the appropriate growth conditions. Membrane vesicles bearing the DtpT-His₆ protein (containing a C-terminal factor-Xa cleavage site and a six-histidine-tag) showed a Pro-Ala uptake activity that was half that of membranes containing the wild-type protein. The activity in the DtpT-His₆ membrane vesicles increased at least 50 % upon removal of the His₆ tag from the protein. More than 95 % DtpT was solubilized from *L. lactis* membranes in the presence of 1 % (mass/vol.) *n*-dodecyl- β -D-maltoside, and approximately 2 mg DtpT-His₆ was purified by Ni²⁺-chelate affinity chromatography from 100 mg membrane protein. Purified DtpT-His₆ was reconstituted unidirectionally into detergent-saturated formed liposomes, which were prepared from *Escherichia coli* phospholipid and egg phosphatidylcholine; the detergent was removed by adsorption to polystyrene beads. The highest uptake activities were obtained when DtpT was incorporated into liposomes that were treated with a low amount of *n*-dodecyl- β -D-maltoside (onset of liposome solubilization). The uptake activity could be improved by addition of NaCl (200 mM) and lipids (2 mg/ml) during the solubilization, purification and reconstitution steps.

Keywords: peptide transport; membrane reconstitution; proteoliposome; amplified expression.

The dipeptide and tripeptide transport protein (DtpT) of *Lactococcus lactis* encompasses a single polypeptide of 463 amino acid residues (Hagting et al., 1994). DtpT is similar to the eukaryotic proton-motive-force-dependent peptide-transport proteins of the PTR family (Steiner et al., 1995) and a peptide-transport protein of *Lactobacillus helveticus* (Nakajima et al., 1997). Examination of the hydropathy profile and analysis of its membrane topology, using the activities of DtpT-reporter fusion proteins and the pattern of labeling of single cysteine mutants by membrane-impermeable sulfhydryl reagents, suggests that the structure of DtpT corresponds to a bundle of 12 α helices with the N and C termini located intracellularly (Hagting et al., 1997).

To further study the structure/function relationships of DtpT, it was necessary to purify the protein and to develop methods for efficient reconstitution into artificial membranes. In these proteoliposomes, the ionic composition on both sides of the membrane is determined by the experimental design, and peptide transport is not influenced by hydrolysis of the substrate. The large-scale purification of (membrane) proteins is highly facilitated by overexpression of the polypeptides, for which *E. coli* is often the preferred host. However, the organism has been

most successful for the amplification of endogenous membrane proteins (homologous expression); the sugar-transport proteins LacY, AraE, XylE, FucP, MelB and GalP, the proline-transporter PutP, and the Na⁺/H⁺ antiporter NhaA have been expressed to levels exceeding 5 % of total membrane protein (Poolman and Konings, 1993; Pourcher et al., 1995). The factors that determine the successful amplification of membrane proteins are largely unknown, but an obvious complication, compared with the overexpression of soluble proteins, relates to the proper membrane insertion and correct folding of the polypeptide when membranes are used as starting material for the isolation of the protein. When membrane insertion and subsequent folding are rate determining in the expression, it is not useful to increase transcription and/or translation. It has been observed that in some *E. coli* mutants, in which the rate of transcription is lower, several membrane proteins are successfully overproduced and inserted into the cytoplasmic membrane, while expression of these membrane proteins in wild-type *E. coli* is lethal (Miroux and Walker, 1996). However, many (heterologous) membrane proteins are toxic to *E. coli* cells, even when expressed at low levels. The deleterious effects probably arise from misfolded material that becomes associated with the cytoplasmic membrane. Overexpression can lead to the formation of inclusion bodies, which are generally less toxic or non-toxic, but denaturation and refolding of the protein are required prior to functional analysis.

The overexpression of membrane proteins in a homologous system may have some advantages over heterologous expression, even though very few studies have addressed this issue.

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Abbreviations. AMS, 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid; DodGlc₂, *n*-dodecyl- β -D-maltoside; MPB, 3-(*N*-maleimidylpropionyl)biocytin.

In the present paper, we report the amplified expression in *L. lactis*, purification and functional reconstitution of the dipeptide/tripeptide-transport protein DtpT. After optimization of the expression levels, choice of detergent and protein-stabilizing agents for solubilization, the His₆-tagged DtpT protein was purified in a single step by nickel-chelate affinity chromatography.

Bacterial strains and growth conditions. *E. coli* strains MC1061 (*Alac*[IPOZYA], *araD*139, Δ [*ara-leu*]7697, *galU*, *galK*, *rspL*, $r_k m_k$) and CJ236 (*dut*, *ung*, *thi*, *relA*, *pCJ105* [Cm^R]) were grown at 37°C, with vigorous aeration, in Luria broth, supplemented with carbenicillin (50 µg/ml) when appropriate. *L. lactis* strains MG1363 and AG300 (MG1363, Δ *dtpT*) were grown at 28°C in M17 (Difco) or in chemically defined medium (medium A; Poolman and Konings, 1988), both at pH 6.4, and supplemented with 0.5% (mass/vol.) glucose plus 5 µg/ml erythromycin (when carrying plasmid vector).

Molecular cloning. Molecular-cloning techniques were performed essentially as described by Sambrook et al. (1989). Plasmids from *L. lactis* were isolated as described (Leenhouts et al., 1990). *L. lactis* was transformed by electroporation as described (Holo and Nes, 1989). All plasmids used in this study are listed in Table 1.

containing DNA of the resulting plasmid pSKF10 was isolated from *E. coli* CJ236 and served as a template for annealing of the oligonucleotide primer DtpTmlu (5'-GATGTTTCGTTACGC GTTAAAGGAATGA). The resulting plasmid (pSKF10Mlu) was transformed into *E. coli* MC1061, and the presence of the new *Mlu*I site was verified by restriction analysis. A linker, consisting of the two annealed oligonucleotides His-linkS (5'-CGC-GATTGAGGGTCGTCATCATCACCACCATCACTGA) and His-linkA (5'-CGCGTCAGTGATGGTGGTGATGATGACGAC-CCTCAAT), that specify the factor-Xa cleavage site and a His₆ tag, were ligated into the *Mlu*I site of pSKF10Mlu. The insert and flanking regions were verified by nucleotide sequencing. The *Kpn*I and *Sal*I sites were used to isolate the 1603-bp fragment, which was exchanged for the corresponding fragment of pDT3, yielding pDT3HT. For expression of the His-tagged protein in *L. lactis*, the approximately 3570-bp *Nco*I–*Sal*I fragment of pDT3HT was isolated and exchanged for the wild-type fragment of pGKF5, yielding pGKHT.

Right-side-out membrane vesicles and proteoliposomes. Right-side-out membrane vesicles or proteoliposomes were fused with cytochrome-*c*-oxidase-containing liposomes by a freeze/thaw step and extrusion through 400-nm polycarbonate filters (Driessen et al., 1985; Mayer et al., 1986). The fused membranes were collected by centrifugation (280 000×*g*, 15 min), and suspended at 1 mg protein/ml in 50 mM potassium phosphate, pH 6.5, 5 mM MgSO₄. The concentrated membrane suspension was diluted 20-fold into oxygen-saturated 50 mM potassium phosphate, pH 6.5, 0.2 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine 20 μM cytochrome *c*. Pro-[¹⁴C]Ala (40 μM) was added 1 min after the addition of 10 mM ascorbate; the reaction mixture was gently stirred while water-saturated air was flushed over the solution. Further handlings were the same as described for transport assays in whole cells, except that 0.45-μm cellulose nitrate filters were used.

Expression levels and raising of antibodies. The amount of DtpT in the samples was estimated by immunodetection with antibodies raised against DtpT-His₆. To raise the antibodies, a rabbit was injected subcutaneously with about 100 µg purified DtpT-His₆ in Freund's adjuvant. The rabbit was boosted after 2 weeks with about 100 µg of the same preparation, and bled 8–12 days after each injection. The antibodies were used at 1:50 000 dilutions. For the western blot analyses, *L. lactis* cells were grown in M17 broth or medium A, supplemented with 0.5% glucose plus 5 µg/ml erythromycin, harvested in the late exponential phase of growth, washed once with water, and suspended in water to A₆₆₀ of 10. The cells were sonicated three times for 5 s on ice, and after addition of sample buffer, boiled for 10 min. The proteins (20 µg/lane) were separated by SDS/

Table 1. Plasmids used. Carb^R and Em^R indicate resistance to carbenicillin and erythromycin, respectively.

Plasmid	Relevant characteristics	Source/reference
pSKII(+)	pBluescript II SK ⁺ , Carb ^R , high-copy expression vector	Stratagene
GKV210	Em ^R , <i>E. coli</i> / <i>L. lactis</i> shuttle vector	Van der Vossen et al. (1985)
pGKF5	pGKV210, containing <i>dtpT</i> of <i>L. lactis</i> on a 4.2-kb <i>EcoRI</i> – <i>Sall</i> fragment	Hagting et al. (1994)
pTAQI	pBR322, Carb ^R , <i>lacI</i> behind penicillinase promoter, expression vector with <i>tac</i> promoter	Genencor Int.
pDT3	pTAQI, carrying <i>dtpT</i> gene of <i>L. lactis</i> on a 3.5-kb <i>HindIII</i> – <i>Sall</i> fragment	Hagting et al. (1994)
pSKF10	pSKII(+), carrying 3' end of <i>dtpT</i> of <i>L. lactis</i> on a 1566-bp <i>KpnI</i> – <i>Sall</i> fragment	this work
pSKF10Mlu	pSKF10, with <i>MluI</i> site at the stop codon of <i>dtpT</i>	this work
pSKF10HT	pSKF10Mlu with linker coding for a factor-Xa cleavage site and a His ₆ tag ligated in the <i>MluI</i> site	this work
pDT3HT	pDT3 with <i>dtpT-H6</i> rather than <i>dtpT</i>	this work
pGKHT	pGKF5 with <i>dtpT-H6</i> rather than <i>dtpT</i>	this work
pGKHT(T360C)	pGKF5 with <i>dtpT(T360C)-H6</i>	Hagting et al. (1997)

PAGE (12 % polyacrylamide) and transferred to poly(vinylidene difluoride) (Millipore) sheets by semidry electroblotting (Kyhse-Anderson, 1984). Detection, by means of the Western-Light chemiluminescence-detection kit with CSPD as a substrate, was performed as recommended by the manufacturer (Tropix Inc.).

Factor-Xa treatment. To make the His₆ tag accessible for the protease from the inside and the outside of the membrane, right-side-out membrane vesicles (500 µg protein in 20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 2 mM CaCl₂) were sonicated three times for 5 s on ice in the presence of 2.5 µg factor Xa. The mixture was incubated for 5 h at 23 °C, and the membrane vesicles were collected by centrifugation (280 000×*g*, 15 min), and suspended in 100 mM potassium phosphate, pH 6.5, 5 mM MgSO₄.

Solubilization. Membrane vesicles (4 mg/ml) were solubilized in 50 mM potassium phosphate, pH 8.0, 400 mM NaCl, 10 % (by vol.) glycerol, plus 1 % (mass/vol.) *n*-dodecyl-β-D-maltoside (DodGlc₂), unless indicated otherwise in the legends to the figures. The mixture was incubated on ice for 20 min, and the insoluble material was removed by centrifugation (280 000×*g*, 15 min).

Purification of DtpT-His₆. The solubilized membrane proteins were mixed with Ni²⁺-nitrilotriacetate resin (about 0.5 mg DtpT-His₆/ml resin) equilibrated with 50 mM potassium phosphate, pH 8.0, 400 mM NaCl, 10 % (by vol.) glycerol, 2 mg/ml *E. coli* lipids, and 0.1 % (mass/vol.) DodGlc₂ (buffer A), unless indicated otherwise. The mixture was incubated for 45 min at 4 °C under continuous shaking, and subsequently poured into a column. The column was washed with 5 column vol. buffer A, followed by 5 column vol. buffer A supplemented with 15 mM imidazole. The protein was eluted with buffer A containing 100 mM imidazole and immediately used for reconstitution. All handlings were performed at 4 °C.

Reconstitution of DtpT-His₆ in proteoliposomes. Reconstitution was performed essentially as described by Knol et al. (1996). A 3:1 mixture of purified *E. coli* total lipid extract (Kawaga et al., 1971) and egg yolk phosphatidyl choline was suspended in 50 mM potassium phosphate, pH 7.0, at 20 mg lipid/ml. Unilamellar vesicles with relatively homogenous size were made by dialysis of octylglucoside-dissolved lipids, followed by freezing in liquid nitrogen, slow thawing at room temperature, and extrusion through a 400-nm polycarbonate filter. The liposomes were diluted to 4 mg phospholipid/ml and titrated with varying amounts of DodGlc₂ and incubated for 1 h at 20 °C. The solubilization of the liposomes was followed by measuring the absorbance at 540 nm (Rigaud and Pitard, 1995). The detergent-treated liposomes were mixed with purified DtpT-His₆ at 100:1 (by mass) and incubated for 30 min at 20 °C under gentle agitation, after which the detergent was removed by absorption to

polystyrene beads (extensively washed with methanol and water; BioBeads SM-2, 80 mg/ml), for 2 h at 20 °C, followed by 2 h and overnight at 4 °C; for each period the polystyrene beads were replaced with new beads. The proteoliposomes were washed with 50 mM potassium phosphate, pH 7.0, concentrated by centrifugation, and stored in liquid nitrogen.

Orientation of the reconstituted DtpT protein. The mutant protein [Cys360]DtpT-His₆ was purified and reconstituted as described above. The proteoliposomes (0.1 mg/ml protein) were treated with 200 µM AMS or untreated for 30 min at 30 °C. The membranes were washed twice (centrifugation for 15 min at 280 000×*g*), suspended to 0.1 mg/ml protein in 50 mM potassium phosphate, pH 7.0, and incubated for 30 min at 30 °C with 200 µM 3-(*N*-maleimidopropionyl)biocytin (MPB). For the outside and inside labeling, the proteoliposome samples were sonicated with a microtip at an output of 4 µm (peak to peak) for 4 s in the presence of MPB. The reactions were stopped by the addition of 10 mM dithiothreitol, and analyzed by western blotting and detection with streptavidin/alkaline phosphatase and CSPD as a substrate.

Miscellaneous. Protein was assayed according to Lowry et al. (1951) with bovine serum albumin as a standard.

Materials. [¹⁴C]Alanine was obtained from Amersham, factor Xa from Biolabs, Ni²⁺-nitrilotriacetate resin from Qiagen, Inc., BioBeads SM2 from Bio-Rad; *n*-octyl-β-D-glucopyranoside and Triton X-100 from Boehringer Mannheim; DodGlc₂ from Sigma; and dodecylglycol from Fluka Chemie AG. Total *E. coli* lipids and L-α-phosphatidylcholine from egg yolk were obtained from Avanti Polar lipids. All other materials were reagent grade and obtained from commercial sources.

RESULTS

Overexpression in *L. lactis*. For amplified expression, the *dtpT* gene and its promoter regions were placed in the *E. coli*/*L. lactis* shuttle vector pGKV210, and the resulting plasmid pGKF5 was used to transform *L. lactis* MG1363 and *L. lactis* AG300 (*AdtpT*). The cells were grown in M17 broth or medium A. The initial rate of uptake of Pro-Ala in whole cells, at a substrate concentration of 40 µM, was 0.5 nmol · min⁻¹ · mg protein⁻¹ for MG1363/pGKV210 grown in M17 broth (Fig. 1A). The introduction of plasmid pGKF5 (multiple copies of *dtpT*) into this strain led to a ninefold increase of the initial uptake rate. A further 2.5-fold increase of the initial uptake rate was observed when the cells were grown in medium A. The transport activity was highest (20 nmol · min⁻¹ · mg protein⁻¹) when pGKF5 was introduced into MG1363, in which *dtpT* was present on the chromosome and the plasmid. To relate uptake activities to expres-

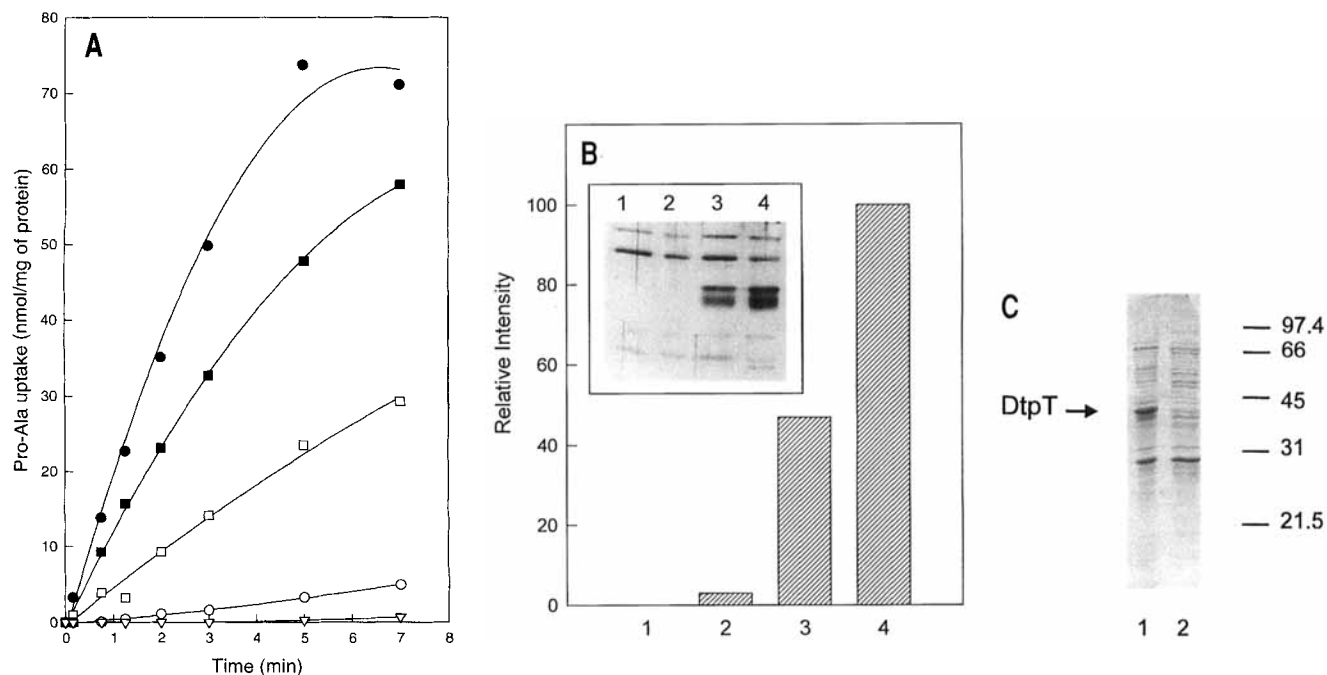


Fig. 1. Effect of growth conditions and gene dosage on Pro-Ala transport (A) and the expression levels of DtpT (B, C) in *L. lactis*. (A) *L. lactis* strains AG300/pGKV210 (▽), MG1363/pGKV210 (○), AG300/pGKF5 (□, ■) and MG1363/pGKF5 (●) were grown in M17 broth (open symbols) or medium A (closed symbols) both supplemented with 0.5 % (mass/vol.) glucose and 5 µg/ml erythromycin. The transport assays in whole cells were performed as described in Materials and Methods at 40 µM Pro-[¹⁴C]Ala. The relative expression levels were estimated from densitometry of the immunoblots (B). Lanes 1–3, AG300/pGKV210, MG1363/pGKV210, AG300/pGKF5, respectively, grown in M17 broth; lane 4, AG300/pGKF5 grown in medium A. The relative amount of DtpT in AG300/pGKF5 cells grown in medium A was set to 100 %. (C) Coomassie-brilliant-blue-stained SDS/PAGE gel (12 %) with samples of urea/cholate-extracted right-side-out membrane vesicles (15 µg protein/lane); lane 1, AG300/pGKF5; lane 2, AG300/pGKV210. The cells used for the preparation of the membrane vesicles were grown in medium A supplemented with 0.5 % (mass/vol.) glucose. The arrow indicates the position of DtpT.

sion levels, the amount of protein in membrane vesicles derived from the various recombinant strains was determined by western blotting (Fig. 1B). Comparison of Fig. 1A and B indicates that the initial uptake rates correlate well with the expression levels of DtpT; the doublet bands observed probably represent different conformations of the (partially unfolded) DtpT protein. The level of DtpT expression in AG300/pGKF5 membrane vesicles was difficult to visualize on Coomassie-brilliant-blue-stained SDS/PAGE gels. After extraction of the membrane vesicles with 5 M urea/6 % (mass/vol.) sodium cholate, the level of DtpT was estimated to be 11 % of total integral membrane protein, as determined by densitometry (Fig. 1C), which is approximately 5 % of crude membrane protein.

Activity of DtpT-His₆. A His₆ tag was constructed at the C-terminus of DtpT to facilitate purification of the protein by immobilized-nickel chelate chromatography (Hochuli et al., 1987). The activity of the His₆-tagged protein was determined in *L. lactis* AG300 cells, harboring pGKHT (His₆-tagged DtpT), and was compared with AG300/pGKF5 (wild-type DtpT). The His₆-tagged protein was functionally expressed with a transport activity of about 50 % that of wild-type DtpT (Fig. 2). Western blot analysis showed that the levels of DtpT-His₆ and DtpT were similar (data not shown); however, small differences in expression levels (20–30 %) are not easily quantitated by the method used.

To test whether the uptake activity of DtpT-His₆ could be restored to wild-type levels upon removal of the His₆ tag, membrane vesicles of AG300/pGKF5 and AG300/pGKHT were treated with factor Xa. Treatment of urea/cholate-extracted vesicles with factor Xa showed a mobility shift, which was complete after 5 h (data not shown), indicating that the His₆ tag was

cleaved off. DtpT-His₆ migrates with a slightly slower mobility than wild-type DtpT as a result of ten additional amino acids at the C-terminus (the factor-Xa cleavage site, IEGR, plus six histidine residues). The uptake of Pro-Ala in AG300/pGKF5 vesicles is not affected by treatment with factor Xa (Fig. 2). The uptake of Pro-Ala in factor-Xa-treated AG300/pGKHT membrane vesicles was significantly higher than in the untreated vesicles. For most of the studies presented below the DtpT-His₆ protein was used.

Solubilization of DtpT. Different concentrations of octyl-β-D-glucoside (1–4 %), DodGlc₂ (0.2–1.5 %), dodecylglycol (0.5–2 %) and Triton X-100 (0.5–2 %) in 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10 % (by vol.) glycerol were tested for their ability to solubilize DtpT-His₆ from right-side-out membrane vesicles. The best results were obtained with 1 % (mass/vol.) DodGlc₂, which solubilized more than 95 % DtpT-His₆ (Fig. 3). All other detergents and other concentrations of DodGlc₂ tested resulted in a less efficient solubilization (<75 % for octylglucoside, <60 % for Triton X-100, <25 % for dodecylglycol) and/or aggregation of DtpT-His₆. Aggregation was observed on immunoblots as higher-order aggregates of DtpT-His₆, especially when high detergent concentrations were used (data not shown). The pH during solubilization was kept at 8.0, since the protein was subsequently used to bind to the Ni²⁺-nitrilotriacetate resin. Lowering of the pH to 7.5 or 7.0 resulted in a slightly less efficient solubilization with 1 % (mass/vol.) DodGlc₂ as detergent. Solubilizations were also carried out with salt concentrations from 0 to 600 mM NaCl but the degree of solubilization was not much affected by the differences in ionic strength.

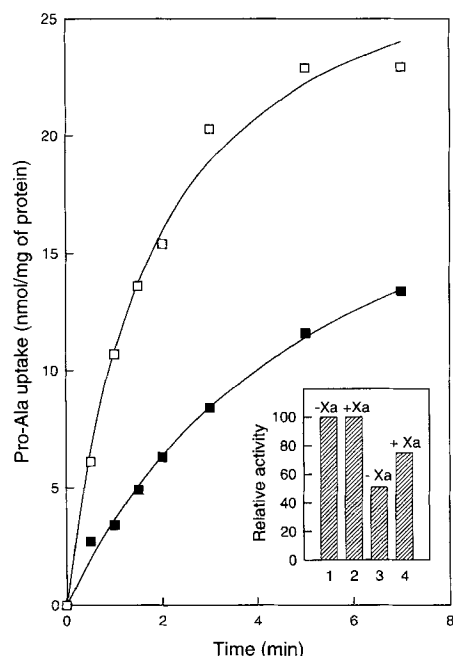


Fig. 2. Activity of DtpT-His₆. *L. lactis* AG300 cells, harboring plasmids pGKF5 (□) and pGKHT (■), were grown in medium A supplemented with 0.5% (mass/vol.) glucose. Pro-Ala uptake was assayed at 25 μ M Pro-[¹⁴C]Ala. The inset shows the relative activities of Pro-Ala uptake in right-side-out membrane vesicles prepared from AG300/pGKF5 (1 and 2) and AG300/pGKHT (3 and 4). The right-side-out membrane vesicles were incubated for 5 h at 23°C without (–) or with (+) factor Xa. The activity of right-side-out AG300/pGKF5 membrane vesicles incubated without factor Xa was taken as 100%; the standard deviations in the activity measurements were less than 10%.

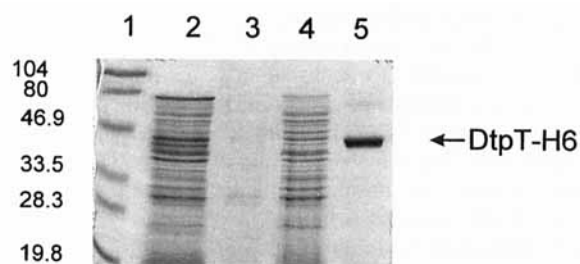


Fig. 3. Purification of DtpT-His₆, as analysed by SDS/PAGE stained with Coomassie Brilliant Blue. A 12% SDS/PAGE gel was loaded with the following samples: molecular-mass markers (lane 1); supernatant (15 min at 280 000 \times g) after solubilization of the membrane vesicles with 1% (mass/vol.) DodGlc₂ (lane 2); pellet after solubilization (lane 3); wash of Ni²⁺-nitrilotriacetate column with buffer A plus 15 mM imidazole (lane 4); and elution with 100 mM imidazole (lane 5).

Purification and reconstitution. Solubilization of DtpT-His₆ from membranes of *L. lactis*/pGKHT for 20 min with 1% (mass/vol.) DodGlc₂ in 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol was used in further studies, which involved purification of the protein by Ni²⁺-chelate affinity chromatography. Most contaminants were removed by washing of the column with 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol, 0.1% (mass/vol.) DodGlc₂ and 15 mM imidazole. The protein was eluted by raising the imidazole concentration to 100 mM. Samples of solubilized membrane vesicles and purified protein were analyzed by SDS/PAGE (Fig. 3) and showed that DtpT-His₆ could be isolated with a purity of greater than 90%. The yield of the procedure was typically 200 μ g DtpT-His₆ protein from 10 mg crude mem-

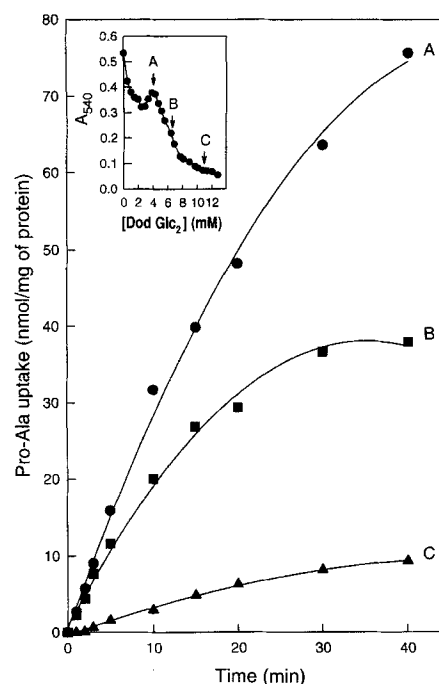


Fig. 4. Pro-[¹⁴C]Ala uptake activity of membrane-reconstituted DtpT-His₆. Purified DtpT-His₆ in buffer A plus 0.1% (mass/vol.) DodGlc₂ was reconstituted in liposomes that were equilibrated for 1 h at 20°C with 3.8 (●), 6.4 (■) and 10.8 mM of DodGlc₂ (▲). The state of the liposomes, followed by measurement of the absorbance at 540 nm, 1 h after addition of DodGlc₂ is shown in the inset; the arrows indicate the three states of solubilization that were used for the reconstitution.

Table 2. Effect of substrate, lipids and salt on the activity of reconstituted DtpT-His₆. The additions were: 1 mM Pro-Ala, 2 mg/ml lipids [*E. coli* total lipid extract and egg yolk phosphatidylcholine at a ratio of 3:1 (by mass)], 200 mM NaCl. The components were present during the solubilization and purification of DtpT. The uptake assay was performed with 40 μ M Pro-[¹⁴C]Ala. Pro-[¹⁴C]Ala uptake was driven by a proton-motive force that was generated by the oxidation of ascorbate/*N,N,N',N'*-tetramethyl-*p*-phenylenediamine/cytochrome *c* via cytochrome-*c* oxidase. The variations in the initial rates were maximally \pm 30% when data from independent experiments were compared, which means that the effect of Pro-Ala is not significant. The standard buffer used for purification and reconstitution was: 50 mM potassium phosphate, pH 8.0, 200 mM NaCl, 10% (by vol.) glycerol and 0.1% (mass/vol.) DodGlc₂.

Addition	Initial rate of uptake
	nmol \cdot min ⁻¹ \cdot mg protein ⁻¹
None	0.8
Pro-Ala	1.2
Lipids	1.9
Pro-Ala plus lipids	2.1
NaCl	2.8
Pro-Ala, lipids and NaCl	3.5

brane vesicles. The purified protein was used to raise antibodies in rabbit.

DtpT-His₆ was immediately reconstituted after purification by mixing the purified protein with liposomes prepared from *E. coli* phospholipids and egg phosphatidylcholine. It has been shown that membrane reconstitution can be highly dependent on the physical state of the lipid/detergent mixture, with the two extremes being the lamellar and micellar state (Knol et al., 1996;

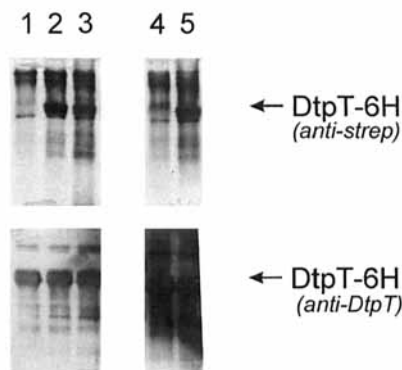


Fig. 5. Labeling of reconstituted [Cys360]DtpT-His₆ with MPB. Proteoliposomes with [Cys360]DtpT-His₆ were treated with AMS (lanes 1 and 4) or untreated (lanes 2, 3 and 5), and labeled with MPB as described in Materials and Methods. The sample shown in lane 3 was sonicated in the presence of MPB to obtain 'maximum' biotinylation of the protein. The samples shown in lanes 4 and 5 represent a duplicate experiment of the one shown in lanes 1 and 2. After labeling, the proteins were subjected to SDS/PAGE, electroblotted, and visualized by streptavidin-conjugated alkaline phosphatase (anti-strep) or anti-DtpT polyclonal Ig; some contaminating proteins are visible upon overnight exposure of the film.

Rigaud et al., 1988). To follow the physical state of the liposomes, the absorbance at 540 nm was measured upon addition of different amounts of detergent (Fig. 4). At three stages of solubilization of liposomes, purified DtpT-His₆ was added, i.e. at the onset of solubilization (liposomes saturated with detergent), halfway through the breakdown of the liposomes, and at the point that the liposomes are fully solubilized (micellar state). For DodGlc₂ these points correspond to 3.8, 6.4 and 10.8 mM, respectively (Fig. 4). Following equilibration of the lipid/detergent mixture with DtpT-His₆, the detergent was removed by adsorption to polystyrene beads. The proteoliposomes were fused with cytochrome-*c*-containing liposomes to generate a proton motive force to drive peptide uptake for prolonged periods of time. The uptake of Pro-[¹⁴C]Ala by DtpT-His₆ reconstituted at the three points in the DodGlc₂ titration curve is shown in Fig. 4. The highest activity was obtained when liposomes were titrated with a low detergent concentration. Using liposomes that were partially desintegrated, the activity was reduced by about 35 %, while the activity was reduced by about 90 % when mixed micelles of lipids and detergent were used.

The uptake activities in the proteoliposomes were not only dependent on the physical state of the liposomes at the beginning of the reconstitution, but also on the NaCl, lipid and substrate concentrations used during the purification and reconstitution (Table 2). The presence of lipids (2 mg/ml) and NaCl (400 mM) during the solubilization, purification and reconstitution increased the initial uptake rate twofold and threefold, respectively. Less important seemed to be the presence of 1 mM Pro-Ala (carrier substrate) or glycerol, which is frequently used to stabilize membrane proteins in their solubilized state (Maloney and Ambudkar, 1989).

Orientation of reconstituted DtpT protein. The orientation of DtpT-His₆ in proteoliposomes was assessed by monitoring the accessibility of the single cysteine in [Cys360]DtpT-His₆ for a membrane-impermeable sulfhydryl reagent. Previous studies on the membrane topology of the DtpT protein have shown that Cys360 is present in a loop region at the outer surface of the membrane (Hagting et al., 1997). If [Cys360]DtpT-His₆ is reconstituted in an inside-in orientation, Cys360 should react with the membrane-impermeable sulfhydryl reagent AMS, which should

protect the protein from subsequent labeling with MPB (Loo and Clarke, 1995). MPB is weakly membrane permeable and upon prolonged incubation and at relatively high concentrations it will react with cysteines that are facing the inner surface of the proteoliposomes. The experiments indicate that [Cys360]DtpT-His₆ was labeled by MPB and the labeling could not be enhanced by sonication of the samples (Fig. 5). Treatment of the proteoliposomes with AMS completely blocked the MPB labeling (Fig. 5), which strongly suggests that the protein is reconstituted with the cytoplasmic surface facing inwards, i.e., the orientation is the same as in the intact cell. The bottom panel of Fig. 5 shows an immunoblot with an anti-DtpT Ig, which indicates that equal amounts of [Cys360]DtpT-His₆ were present in each lane.

DISCUSSION

In this paper we describe the amplified expression, purification and functional reconstitution of the dipeptide/tripeptide-transport protein of *L. lactis*. The expression of the DtpT protein was increased by using its own expression signals (promoter and Shine-Dalgarno sequences) and exploiting a convenient low-copy *L. lactis*/*E. coli* shuttle vector. We show that engineering of an affinity tag to the protein and use of nickel-chelate affinity chromatography provide an efficient and simple means to separate the carrier protein from (most of) the other membrane proteins. Reconstitution of the purified DtpT protein into detergent-destabilized liposomes, was unidirectional and highly reproducible.

Cell death (lysis) is frequently observed upon overproduction of heterologous membrane proteins in *E. coli* (Grisshammer and Tate, 1995). Overexpression of DtpT in *E. coli* was in most cases lethal (unpublished results). However, by means of a homologous expression system the problems of cell death could be avoided and rather high levels of expression were obtained. This is probably due to a better tuning of the rate of transcription/translation to the rate of membrane insertion and folding, which leads to higher amounts of functional protein in the membrane. As observed for the ATP-driven peptide-transport systems (Opp and DtpP) of *L. lactis*, the expression driven by the endogenous promoter was dependent on the composition of the growth medium. For DtpT, medium A without peptides yielded the highest level of expression. A similar observation has been made for the oligopeptide-transport system Opp (Detmers, F. M., unpublished results), whereas the DtpP system is induced by dipeptides and tripeptides containing branched-chain amino acids (Foucaud et al., 1995).

For the reconstitution of transport proteins, *n*-octyl- β -D-glucopyranoside solubilization of membrane vesicles and detergent removal by dilution or dialysis is often used (Baron and Thompson, 1975). This procedure has in many cases been improved by the addition of phospholipids and osmolytes (often glycerol), which seem to stabilize the proteins when they are in the detergent-solubilized state (Newman and Wilson, 1980; Maloney and Ambudkar, 1989; Poolman and Konings, 1993). Although a number of proteins have been reconstituted successfully after solubilization with *n*-octyl- β -D-glucopyranoside, several other proteins are irreversibly inactivated by this detergent (Knol, J., unpublished results). Therefore, we have tested a number of detergents for their ability to solubilize and retain activity of the DtpT protein. With a heterologous expression system this may require other detergents and conditions than when the same protein is purified from its natural host (Grisshammer and Tate, 1995). Very few studies have been performed in which membranes of lactic acid bacteria were used as source to isolate or purify a particular membrane protein. In our studies, we fa-

voured DodGlc₂ for the solubilization, purification and reconstitution of DtpT. With the purpose of reconstituting DtpT unidirectionally (Knol et al., 1996), liposomes were titrated with detergent, and the A₅₄₀ was measured to follow the physical state of the liposomes. For DodGlc₂, the steady-state absorbance values are reached very slowly (> 60 min), which makes it time consuming to follow the solubilization by stepwise addition of DodGlc₂. Different amounts of DodGlc₂ were added to different portions of liposomes and the A₅₄₀ was determined after 1 h of incubation at 20 °C (Fig. 4). At this time-point, the system has not been equilibrated fully, but it offers a good diagnostic measure of the physical state of the liposomes. These conditions were studied most extensively for the reconstitution of DtpT-His₆. With other detergents, such as dodecyloctaglycol or Triton X-100, the steady-state-absorbance values were achieved rapidly (< 2 min), but the reconstitution of DtpT in these liposomes resulted in lower uptake activities (data not shown).

The advantage of reconstituting membrane proteins into detergent-destabilized liposomes might be that one has better control of the incorporation of the protein into the bilayer and that the protein is faced with lower detergent concentrations. It has been suggested that the insertion of a protein into liposomes leads to proteoliposomes with better unidirectional protein insertion than when proteoliposomes are formed by coalescence of mixed micelles containing lipid, protein and detergent (Eytan, 1982; Helenius et al., 1981). A possible explanation for the unidirectional reconstitution of proteins into liposomes may be found in the difference in hydrophobicity between the outer and inner surfaces of the protein (Rigaud and Pitard, 1995). For secondary transport proteins the cytoplasmic loops that connect the transmembrane-spanning segments are usually much longer than the outer loops (Poolman and Konings, 1993), and therefore the cytoplasmic surface can be more hydrophilic, which would favour an inside-out reconstitution, as was observed for the lactose-transport protein (LacS) of *S. thermophilus* (Knol et al., 1996). For DtpT we observed the opposite, i.e. the protein was reconstituted in an inside-in orientation (Fig. 5), which suggests that factors other than surface hydrophilicity affect the directed insertion of the DtpT protein into detergent-destabilized liposomes.

The highest uptake activities of DtpT were observed when reconstitution was performed with liposomes that were fully saturated with detergent but still in the lamellar state (onset of solubilization), which could relate to the unidirectional incorporation under these conditions assuming that the kinetic parameters of transport are different for the protein in the inside-out and inside-in orientations. Since, the transport assays were performed at a substrate concentration far below the $K_m^{app}(out)$, a higher $K_m^{app}(in)$ compared with $K_m^{app}(out)$ would be reflected in a lower uptake rate if part of the carriers were reconstituted inside-out. On the other hand, we cannot exclude that the amount of (functionally) reconstituted protein is different under the various conditions tested or that high concentrations of DodGlc₂ inactivate DtpT-His₆.

The initial uptake rate of Pro-Ala by DtpT-His₆ in whole cells was 4 nmol · mg protein⁻¹ · min⁻¹ (Fig. 2). A decrease in specific activity was observed when right-side-out membrane vesicles, prepared from cells expressing the His₆-tagged DtpT, were used to study peptide transport. However, in whole cells Pro-Ala is transported down the concentration gradient, because the dipeptide is immediately hydrolyzed. The lower specific uptake activity in the membrane vesicles and proteoliposomes may have been caused by substrate inhibition at the inner surface and/or a lower proton-motive force (and internal pH). The engineering of a His₆ tag at the C terminus of DtpT decreased the uptake of Pro-Ala in whole cells by about 50%. A similar phe-

nomenon observation was made for His₆-tagged Tn10-specified tetracycline efflux antiporter TetA (Aldema et al., 1996). Removal of the His₆ tag from the DtpT fusion protein led to an increase of the activity. However, the specific activity of purified reconstituted DtpT-His₆ is sufficient to study the specificity and kinetics peptide transport in detail, and allows us to evaluate the properties of any mutant DtpT protein in an artificial membrane system.

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